Antigen receptor triggering and apoptotic pathways in neoplastic B cells
Mackus, W.J.M.

Link to publication

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

UvA-DARE is a service provided by the library of the University of Amsterdam (http://dare.uva.nl)
CHAPTER 4

B cell receptor triggering reduces apoptosis in B-CLL patients with unmutated IgV<sub>H</sub> genes and ZAP-70 expression

SUBMITTED FOR PUBLICATION

Wendelina J.M. Mackus<sup>1,2</sup>
Annette Grummels<sup>1,2</sup>
Ludo M. Evers<sup>1</sup>
Richard J. Bende<sup>3</sup>
Mark H.H. Kramer<sup>4</sup>
Eric Eldering<sup>2</sup>
René A.W. van Lier<sup>2</sup>
Marinus H.J. van Oers<sup>1</sup>

<sup>1</sup>Dept. of Hematology,
<sup>2</sup>Laboratory for Experimental Immunology, and
<sup>3</sup>Dept. of Pathology, Academic Medical Center,
University of Amsterdam, and
<sup>4</sup>Dept. of Internal Medicine, Meander Medical Center, Amersfoort, The Netherlands
Chapter 4

ABSTRACT

Recent evidence suggests that B cell chronic lymphocytic leukemia (B-CLL) comprises two disease entities. Patients with malignant B cells that carry unmutated IgV_H genes and express the protein tyrosine kinase ZAP-70 generally have a poor prognosis, whereas an indolent clinical course is common in most patients with mutated IgV_H genes and no expression of ZAP-70 in their malignant B cell clone. Since the ZAP-70 kinase is involved in signal transduction following antigen receptor ligation, we have investigated whether differential functional outcome after BCR triggering could account for the distinct biological behavior of the B-CLL subgroups. Also in our cohort, the presence of unmutated IgV_H genes correlated with protein expression of ZAP-70. CLL B cells from both subgroups (8 patients with unmutated and 18 patients with mutated IgV_H genes) did not differ with respect to spontaneous apoptosis or cytostatic drug-induced apoptosis in vitro. However, in patients with unmutated IgV_H genes BCR ligation resulted in reduced spontaneous apoptosis. In addition, BCR triggering reduced the efficacy of cytotoxic drug-induced cell death in unmutated IgV_H but not in mutated IgV_H B-CLL. These findings might explain the unfavorable clinical outcome in unmutated IgV_H B-CLL patients.

INTRODUCTION

B cell chronic lymphocytic leukemia (B-CLL) is characterized by a clonal expansion of CD5^+CD19^+CD23^++ B cells in the blood, bone marrow and secondary lymphoid tissues and has a heterogeneous clinical course.\cite{48,212}

Based on analysis of mutations in the immunoglobulin heavy chain variable (IgV_H) genes two subgroups of B-CLL patients can be distinguished.\cite{55,56} B-CLL patients with unmutated IgV_H genes have a shorter median survival than patients with extensive IgV_H gene mutations.\cite{55,56} Recently, gene expression profiles of both subgroups of CLL B cells have been shown to be most similar to those of memory B cells.\cite{56} Interestingly, the presence of unmutated IgV_H genes has been associated with expression of a subset of genes which are preferentially activated upon triggering of the B cell receptor (BCR), the so-called “BCR activation gene signature” .\cite{59} Additionally, the mRNA encoding for zeta-associated protein 70 (ZAP-70) was found to be strongly expressed in the unmutated IgV_H patients only.\cite{59}

ZAP-70 is a protein tyrosine kinase (PTK) which is normally expressed in T cells and natural killer cells and is involved in signal transduction via the T cell receptor.\cite{56,67} This kinase is related to the PTK Syk, which, although expressed in all hematopoietic cells, is required for development and clonal expansion of mature B cells.\cite{91,92} The BCR complex is a hetero-oligomeric complex consisting of an antigen-binding cell-surface immunoglobulin coupled to a heterodimer of CD79a (Igα) and CD79b (Igβ). Upon engagement of the BCR the immunoreceptor tyrosine-based activation motifs (ITAMs) of CD79α and CD79β become phosphorylated by the src-kinase Lyn thereby serving as docking sites for Src Homology-2 (SH2) domains present in Syk.\cite{214} In a similar way, ZAP-70 is recruited to the activated TCR via binding of its SH2-domains to the phosphorylated ITAMs of the zeta chains of the TCR.\cite{86} Further downstream signaling is mediated by the subsequent activation and phosphorylation of Syk and ZAP-70 themselves. In agreement with the cDNA microarray data, two recent
studies showed that ZAP-70 protein is predominantly expressed in leukemic B cells of unmutated IgV_H B-CLL patients. Following BCR ligation, CLL B cells of this subgroup exhibited significantly increased tyrosine phosphorylation of cytosolic proteins, including ZAP-70 and Syk, and association of ZAP-70 with the proteins of the BCR complex.

Considering the findings that (1) following BCR ligation ZAP-70 is phosphorylated and associated to the BCR complex in B cells expressing unmutated IgV_H genes and (2) these B-CLL patients have a poor prognosis compared to B-CLL patients with mutated IgV_H genes, we here investigated the functional consequences of BCR activation within the two groups of B-CLL patients. Our data show that only in patients with unmutated IgV_H genes BCR activation prevents CLL B cells from both spontaneous and cytostatic drug-induced apoptosis. These findings might be of importance for understanding the difference in biological behavior of the two subgroups of B-CLL.

PATIENTS, MATERIAL AND METHODS

Patient characteristics and cell isolation
Peripheral blood samples were obtained from 26 B-CLL patients from the outpatient clinic of the department of Hematology of the Academic Medical Center, Amsterdam, and the department of Internal Medicine, Meander Medical Center, Amersfoort, The Netherlands. All individuals gave written informed consent and the study was approved by the local Ethical Review Board. Clinical characteristics of patients are presented in table 1. Patients were staged according to the criteria of Rai et al. The cohort contained 9 patients with Rai stage 0 (mean age in years 68 ± 9, mean ± sd), 10 patients with Rai stage I-II (mean age in years 60 ± 14) and 7 patients with Rai stage III-IV (mean age in years 61 ± 8).

Peripheral blood mononuclear cells (PBMCs) were isolated from diluted EDTA-blood via density gradient centrifugation after which the leukemic cells were purified via negative depletion as described previously. In brief, to remove T cells, monocytes and granulocytes, PBMCs were incubated with saturating amounts of CD3 mAb, CD14 mAb (clone CLB-Mon/1) and CD16 mAb (clone CLB-FcR gran/1) (5 µg/ml, all Sanquin, Amsterdam, The Netherlands) after which cells were incubated with anti-mouse Ig-coated magnetic beads (Dynabeads, M450, Dynal A.S., Oslo, Norway). Bead-coated cells were removed with a Dynal magnetic particle concentrator. The purified leukemic cells contained > 94% of CD5+CD19+ B cells.

Flow cytometry
PBMCs or purified CLL cells were incubated with either fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated mAbs (concentrations according to manufacturer's instructions) against cell surface markers (mAbs used are anti-CD5 (Sanquin), anti-CD19 (Sanquin) and CD23 (Dako A/S, Glostrup, Denmark)) and analyzed by flow cytometry with the Cellquest Program on a FACS Calibur (Becton Dickinson, San Jose, CA) as described.

To measure expression of CD38, cells were stained with 5 µL FITC-labeled anti-CD19 and PE-labeled anti-CD38 (clone T16, Immunoquality Products, Groningen, The Netherlands). The dotplot was gated on the lymphoid gate via the forward scatter (FSC) and sideward scatter (SSC). Cells were further gated on CD19-positive cells after which the percentage of CD38 positive cells was determined using the quadrant marker set according to the negative control which were cells treated with FITC- and PE-labeled isotype control IgG1/IgG2a mAb (Becton Dickinson) with irrelevant specificity. A cut-off point of 30% was used to consider CLL populations to be CD38 positive.

To measure expression of the B cell receptor (BCR) cells were stained for 20 min at 4°C with 5 µL of non-conjugated anti-IgM mAb (clone CLB/MH15, Sanquin) which was also used for
Chapter 4

Table 1. Clinical characteristics of B-CLL patients

<table>
<thead>
<tr>
<th>Pat#</th>
<th>Sex</th>
<th>Age (yrs)</th>
<th>Rai stage</th>
<th>WBC count</th>
<th>Therapy</th>
<th>IgVH status</th>
<th>CD38 (%)</th>
<th>ZAP-70</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>78</td>
<td>0</td>
<td>61.6</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>46</td>
<td>1</td>
<td>12.4</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>84</td>
<td>1</td>
<td>16.9</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>47</td>
<td>2</td>
<td>30.4</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>62</td>
<td>3</td>
<td>103.0</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>73</td>
<td>3</td>
<td>19.3</td>
<td>-</td>
<td>-</td>
<td>90</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>69</td>
<td>4</td>
<td>194.8</td>
<td>+</td>
<td>-</td>
<td>4</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>59</td>
<td>4</td>
<td>52.6</td>
<td>+</td>
<td>-</td>
<td>70</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>69</td>
<td>0</td>
<td>22.5</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>55</td>
<td>0</td>
<td>9.0</td>
<td>-</td>
<td>+</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>73</td>
<td>0</td>
<td>26.0</td>
<td>-</td>
<td>+</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>67</td>
<td>0</td>
<td>36.5</td>
<td>-</td>
<td>+</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>F</td>
<td>82</td>
<td>0</td>
<td>18.6</td>
<td>-</td>
<td>+</td>
<td>9</td>
<td>ND</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>63</td>
<td>0</td>
<td>10.4</td>
<td>-</td>
<td>+</td>
<td>20</td>
<td>ND</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>58</td>
<td>0</td>
<td>80.7</td>
<td>-</td>
<td>+</td>
<td>21</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>F</td>
<td>67</td>
<td>0</td>
<td>18.4</td>
<td>(+)</td>
<td>+</td>
<td>91</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>M</td>
<td>63</td>
<td>1</td>
<td>20.1</td>
<td>-</td>
<td>+</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>M</td>
<td>58</td>
<td>1</td>
<td>24.9</td>
<td>+</td>
<td>+</td>
<td>8</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>M</td>
<td>49</td>
<td>2</td>
<td>88.1</td>
<td>(+)</td>
<td>+</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>M</td>
<td>78</td>
<td>2</td>
<td>10.9</td>
<td>(+)</td>
<td>+</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>21</td>
<td>F</td>
<td>55</td>
<td>2</td>
<td>56.6</td>
<td>-</td>
<td>+</td>
<td>5</td>
<td>ND</td>
</tr>
<tr>
<td>22</td>
<td>M</td>
<td>74</td>
<td>2</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>M</td>
<td>48</td>
<td>2</td>
<td>34.6</td>
<td>-</td>
<td>+</td>
<td>38</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>M</td>
<td>50</td>
<td>3</td>
<td>10.9</td>
<td>-</td>
<td>+</td>
<td>36</td>
<td>ND</td>
</tr>
<tr>
<td>25</td>
<td>M</td>
<td>61</td>
<td>4</td>
<td>247.0</td>
<td>(+)</td>
<td>+</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td>M</td>
<td>54</td>
<td>4</td>
<td>48.8</td>
<td>-</td>
<td>+</td>
<td>55</td>
<td>-</td>
</tr>
</tbody>
</table>

Of 26 patients the following characteristics were determined at time of inclusion in the study; sex (M= male, F= female); age (in years); White Blood Cell (WBC)-count (x 10^3/L); Rai stage: 0 (good prognosis), I-Ill (intermediate prognosis), and III-IV (poor prognosis); therapy (- = no therapy, + = therapy, (+) = prior received therapy which stopped at least 3 months before inclusion); IgVH mutation status (- = unmutated IgVH genes, + = mutated IgVH genes); CD38: the percentage of CD19^CD38^ cells; ZAP-70 protein expression detected by Western blot and/or flow cytometry (- = negative, + = positive); and ND = not determined.

triggering the BCR in the stimulation experiments. After washing the cells were incubated with FITC-labeled goat-anti-mouse mAb for 20 min at 4°C. Cells were washed again with PBS + 0.5% BSA and analyzed by flow cytometry. Both the percentage as well as the mean fluorescence intensity (MFI) of positive cells was determined as cells were gated on the lymphoid gate via FSC/SSC and using the quadrant marker set according to the negative control.

To measure expression of ZAP-70, cells were stained in a multi-step procedure in which all incubations with mAbs were performed on ice for 20 min. First, cells were incubated with PE-labeled anti-CD19 and APC-labeled anti-CD3 (both Becton Dickinson). After washing the cells were fixed in 4% paraformaldehyde (Sigma) for 10 min. Subsequently, cells were washed once
with PBS and twice with permeabilisation solution (PBS + 0.5% BSA + 0.1% saponin (Calbiochem, Darmstadt, Germany)) after which cells were suspended in permeabilisation solution and incubated for 20 min. Cells were then incubated with non-conjugated anti-ZAP-70 mAb (clone 2F3.2, Upstate Biotechnology, Lake Placid, NY). After washing with PBS+ 0.5% BSA cells were incubated with FITC-labeled isotype specific IgG2a goat-anti-mouse mAb (Southern Biotech Association, Birmingham, AL). After a final wash with PBS + 0.5% BSA cells were analyzed by flow cytometry.

V\textsubscript{H} gene analysis

Total cellular RNA was isolated from 10 x 10\textsuperscript{6} purified B-CLL cells using the TRIZOL reagent (Gibco, Life Technology, Paisley, UK) according to manufacturer’s instructions. Complementary DNA (cDNA) was synthesized out of 2 µg RNA using oligo-dT primer and Superscript II RT (Gibco). Of each patient the V\textsubscript{H} gene mutation status was determined by PCR technique as described previously\textsuperscript{218}. In brief, two steps of PCRs were executed, a V\textsubscript{H} family-specific PCR and a complementarity determining region 3 (CDR3)-specific PCR. For the V\textsubscript{H} family-specific-PCR, separate reactions were performed on cDNA with each of the V\textsubscript{H} family-specific leader primers, combined with the reverse Cu\textsubscript{H} primer. To confirm the clonally expressed V\textsubscript{H} family, a second PCR was performed via amplification of the CDR3 using a forward primer with specificity for framework region 3 in combination with reverse primers specific for Cu\textsubscript{H}. Either 1.5 µL of cDNA (for a nested PCR) or 2 µL of PCR product from a V\textsubscript{H} family-specific PCR was used. The specific sequences of the primers and the PCR reaction schedules have been described previously\textsuperscript{218}. Sequencing of the clonal V\textsubscript{H} family PCR product was performed with an ABI sequencer (Perkin Elmer Corp., Norwalk, CT) using the dye-terminator cycle-sequencing kit (Perkin Elmer Corp.), according to manufacturer’s instructions. The sequences found were compared with published germline sequences, using the Vbase database\textsuperscript{219} and DNAplot on the Internet (http://www.mrc-cpe.cam.ac.uk/DNAplot) to identify mutations. Patients were considered to express unmutated IgV\textsubscript{H} genes when <2%\textsuperscript{55,56} of the V\textsubscript{H} family sequence was mutated when compared to the most homologous germline sequence.

Analysis of apoptosis

Viability of cells was analyzed via detection of phosphatidyl serine on the outer membrane of apoptotic cells as described previously\textsuperscript{143}. In brief, cells were harvested and washed in ice-cold HEPES buffer (10 mM HEPES, 150 mM KCL, 1 mM MgCl\textsubscript{2} and 1.3 mM CaCl\textsubscript{2}, pH 7.4) supplemented with 1 mg/ml glucose and 0.5% (w/v) BSA. Cells were then incubated with FITC-labeled Annexin V (APOTEST-FITC, ImmunoQuality Products) (diluted 1:200 in HEPES buffer) for 15 min and washed twice in HEPES buffer. Just before analysis of the samples by flow cytometry (FACS Calibur, Becton Dickinson), propidium iodide (PI) (Sigma, St. Louis, MO) was added (final concentration 5 µg/ml) to distinguish necrotic cells (Annexin V+/PI\textsuperscript{+}) from apoptotic cells (Annexin V\textsuperscript{-}/PI\textsuperscript{+} and Annexin V\textsuperscript{-}/PI\textsuperscript{-}). Upon induction of apoptosis Annexin V-FITC positivity of cells increased at least by 1 log scale.

In the cell stimulation experiments only freshly isolated, purified B-CLL cells were used. Cells were cultured in complete IMDM medium at a concentration of 2.5 x 10\textsuperscript{5} cells/ml in a 48-wells plate. For determination of apoptosis due to in vitro culture cell viability was detected at least in duplicate directly after isolation (t=0) and upon 48 hrs of in vitro culture in complete medium. Next to this, cells were incubated with either one of the following cytostatic drugs at different final concentrations: etoposide, 0 – 10 – 25 – 50 µM (Sigma); chlorambucil, 0 – 10 - 50 – 100 µM (Sigma); and fludarabine, 0 – 10 - 50 –100 µM (Sigma) or with the anti-IgM mAb (CLB/MH15, Sanquin) (5 µg/ml) to trigger the B cell receptor. In addition, in 3 patients expressing unmutated IgV\textsubscript{H} genes and 2 patients expressing mutated IgV\textsubscript{H} genes cells were cultured with cytostatic drugs in the presence of the anti-IgM antibody. In all experiments induction of apoptosis was detected in duplicate after stimulation of cells for 48 hrs. In case of cytostatic drug treatment the concentration by which 50% of cells died by apoptosis (LC\textsubscript{50}; lethal concentration 50%) was determined for each patient.
Chapter 4

Immunoblotting

For the detection of proteins via immunoblotting thawed cells were washed with ice-cold PBS and to check for contamination of the sample with T cells the percentage of CD3+ cells as well as CD5+CD19+ cells was assessed via FACS analysis. Cells (1.0 x 10^7) were suspended in lysis buffer (1% Nonidet P-40, 0.01 M triethanolamine-HCl (pH 7.8), 0.15 M NaCl, 5 mM EDTA, 0.02 mg/ml ovomucoid trypsin inhibitor, 1 mM PMSF, 0.02 mg/ml leupeptin and 25 μM phenylarsine oxide). After 5-15 min on ice, lysates were cleared by centrifugation at 13,000 x g for 15 min. Protein contents were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, München, Germany) and equal protein loading was in all cases confirmed by Ponceau Red staining and by detection of ERK. After transfer to nitrocellulose (Hybond-C, Amerham, Little Chalfont, UK), blots were blocked with 5% non-fat dry milk in TBS-T (10 mM Tris, 150 mM NaCl, and 0.01% Tween-20, pH 8.0). Blots were probed with the anti-ZAP-70 antibody (clone 29,Transduction Laboratories, Lexington, KY) diluted in TBS-T containing 2.5% non-fat dry milk. Immunoreactive proteins were visualized using HRP-conjugated Ig (GαM) and enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, U.K.).

Statistical analyses

Two-sided Mann-Whitney U test was used for analysis of differences between groups whereas for analysis of differences in the proportion of individuals with a specific characteristic between two groups the Fisher's exact test was used. The Wilcoxon signed rank test was used to compare the differences between paired groups. P-values < 0.05 were considered statistically significant.

RESULTS

Clinical and biological characteristics of unmutated and mutated IgV_H B-CLL patients

Of 26 B-CLL patients the IgV_H gene mutation status was analyzed. In 18/26 cases (69 %) ≥ 2% of mutations in the IgV_H gene were detected whereas 8/26 cases (31%) showed unmutated IgV_H genes.

No statistically significant differences between the two groups existed as to either Rai stage, the proportion of patients receiving therapy at time of inclusion, the absolute number of peripheral blood leucocytes, the percentage of lymphocytes and the percentage of CD5+CD19+ as well as the fraction of CD23+ cells. Expression of CD38 was not significantly different between both groups of patients (P = 0.2853). No significant differences in the clinical and biological characteristics were observed between B-CLL patients expressing high or low CD38.

ZAP-70 protein is expressed in B-CLL patients with unmutated IgV_H genes

By cDNA microarray analysis of B-CLL a higher expression of the ZAP-70 gene has been observed in IgV_H unmutated patients when compared to mutated B-CLL patients which was corroborated at the protein level. We monitored ZAP-70 protein expression via Western blot analysis in cells of 13 patients (Fig. 1A, Table 1 and data not shown). Indeed, unmutated IgV_H gene status was associated with strong expression of ZAP-70 protein in 4/4 patients analyzed. In CLL patients with mutated IgV_H genes ZAP-70 was either absent or weakly expressed. To control for possible contaminating ZAP-70-expressing T cells in all experiments a titration curve of the ZAP-70-negative Ramos B cell line mixed with T cells was tested on the same
immunoblot ZAP-70 could be visualized already when a B cell lysate contained 4 to 8% of T cells (Fig. 1A).

Therefore, the weak expression of ZAP-70 detected in some of the mutated IgV<sub>H</sub> patients might be explained by contamination of the sample with T cells. For each sample the percentage of CD3<sup>+</sup> cells was determined via FACS analysis as indicated. Positive ZAP-70 protein expression was reproducibly found upon analysis of lysates of different blood samples of all patients analyzed (data not shown). We did not find a correlation between CD38 and ZAP-70 protein expression (P = 0.2844).

Next, expression of ZAP-70 protein was quantified by immunostaining and flow cytometry (n=15). Fig. 1B shows a representative dotplot of an IgV<sub>H</sub> unmutated (patient 1, lane 2 Fig. 1A) and mutated (patient 26, lane 7 Fig. 1A) B-CLL patient. B cells of 4/5 unmutated IgV<sub>H</sub> patients expressed ZAP-70 whereas no ZAP-70-positive B cells were detected in 9/10 mutated IgV<sub>H</sub> patients. Thus, discordant results were found in two patients: one unmutated IgV<sub>H</sub> patient did not express ZAP-70 and one mutated IgV<sub>H</sub> patient clearly expressed ZAP-70. In all cases (3 unmutated and 7 mutated IgV<sub>H</sub> cases) in which ZAP-70 protein expression was quantified via both Western blot and flow cytometry similar results were obtained. In all patients the small fraction of contaminating T cells displayed high ZAP-70 expression (data not shown). As positive and negative controls Jurkat T cells and Ramos B cells were used respectively (data not shown).
Apoptosis occurring after in vitro culture or after incubation with cytostatic drugs is similar in patients with unmutated and mutated IgV<sub>H</sub> genes

In agreement with earlier observations 108, apoptosis of leukemic cells was observed during in vitro culture. The percentage of apoptotic cells increased over time (t=0: 9 ± 1%; t=48: 51 ± 2% of apoptotic cells, mean ± SEM). No statistically significant difference between the two subgroups of B-CLL in the percentage of apoptotic cells detected immediately after isolation was observed (t=0) (unmutated: 9 ± 2% of apoptotic cells, mean ± sem; mutated: 9 ± 1%, P =0.4902) nor after in vitro culture for 48 hrs (unmutated: 53 ± 4%; mutated: 51 ± 3%, P =0.8538) (Fig. 2).

We studied whether the IgV<sub>H</sub> mutation status might be related to apoptosis observed after incubation of CLL B cells with cytostatic drugs in 25 B-CLL patients. For etoposide the LC<sub>50</sub> was 11 ± 1 µM (mean ± SEM), for chlorambucil 29 ± 5 µM and for fludarabine 25 ± 3 µM. No significant differences in the drug-induced apoptotic response were detected when comparing IgV<sub>H</sub> unmutated with mutated cases (Fig. 3). Thus, the IgV<sub>H</sub> mutation status of B-CLL patients does not seem to correlate with in vitro sensitivity to cytostatic drugs.

BCR triggering can rescue leukemic B cells from apoptosis in B-CLL patients with unmutated IgV<sub>H</sub> genes

B-CLL patients whose leukemic cells have unmutated IgV<sub>H</sub> genes were reported to express a BCR activation gene signature 59. We investigated whether IgV<sub>H</sub> mutation status of CLL B cells is related to apoptotic responses after BCR triggering.
Assessment of 26 B-CLL patients showed that the response after BCR ligation can be very heterogeneous: the percentage of viable cells can either increase, decrease or remain unchanged relative to culture in medium only (referred to as control). Nevertheless, leukemic B cells with unmutated IgV_H genes were rescued from apoptosis by BCR ligation (127 ± 6% of viable cells of control, mean ± SEM; range 96 – 146%). In contrast, BCR-triggering of mutated IgV_H cells resulted in a decrease in the percentage of viable cells (91 ± 7% of viable cells of control, range 13-123%, P = 0.0021) (Fig. 4). The possibility that signaling via Fc receptor engagement by the anti-IgM mAb is responsible for the observed effects of BCR triggering could be excluded. In both B-CLL subgroups blockage of Fc receptors on CLL B cells with anti-CD32 mAb prior to stimulation with the anti-IgM mAb did not affect the observed results of BCR stimulation (data not shown). Also, neither the percentage nor the mean fluorescence intensity of anti-IgM-positive cells differed significantly between IgV_H unmutated and mutated cases (data not shown).

**Figure 4. BCR-responsiveness of leukemic B cells of CLL patients**

Cells were stimulated with the anti-IgM mAb for 48 hrs after which apoptosis of leukemic cells was determined via Annexin V-FITC/PI staining in duplicate. To clearly demonstrate the effect of anti-IgM ligation, the percentage of viable cells after BCR triggering was expressed relative to the percentage of viable cells after culture in medium only, which was 100% and set to 0 in this figure. BCR triggering leads to a significant increase in the percentage of viable cells in unmutated IgV_H patients (filled symbols) when compared to mutated IgV_H patients (open symbols) as indicated with one asterisk, P =0.0226).

BCR triggering reduced sensitivity of leukemic B cells to cytostatic drug-induced apoptosis in patients with unmutated IgV_H genes

Finally, it was investigated whether triggering of the BCR could modify cytostatic drug-induced apoptosis. Cells of 3 unmutated and 2 mutated IgV_H gene B-CLL patients were incubated with different concentrations of either etoposide, chlorambucil or fludarabine in combination with the anti-IgM mAb for 48 hrs. In the unmutated IgV_H patients there was a very clear protective effect of BCR ligation when cells were incubated at low concentrations of cytostatic drugs. Fig. 5 shows the results for a representative unmutated IgV_H patient. Importantly, these low concentrations lie within the range of concentrations achieved in vivo during chemotherapeutical treatment (indicated in Fig. 5). At higher concentrations of cytostatic drugs this effect was no longer evident. In contrast to the IgV_H mutated subtype, in patients expressing unmutated IgV_H genes BCR triggering resulted in an increase in LC_{50} for each of the cytostatic drugs. In contrast, in mutated IgV_H patients the LC_{50} was unaffected (Fig. 6). These data indicate that in IgV_H unmutated patients BCR triggering reduces the sensitivity of leukemic cells to undergo cytostatic drug-induced apoptosis.
Figure 5. BCR triggering reduces sensitivity of leukemic cells to cytostatic drug-induced apoptosis in B-CLL patients with unmutated IgV_H genes
Cells were incubated for 48 hrs with either etoposide (A), chlorambucil (B) or fludarabine (C) alone (filled squares) or in combination with anti-IgM mAb (open squares) after which the percentage of apoptosis was detected by Annexin V-FITC/PI staining in duplicate. In each graph, the range of in vivo concentrations achieved upon chemotherapeutic treatment is indicated with a grey area which for etoposide is 1.6 - 2.7 μM^{23}, for chlorambucil 1.0 - 2.7 μM^{24}, and for fludarabine 0.4 - 0.6 μM^{26}. Data represent one out of three patients analyzed, showing similar results.

Figure 6. BCR triggering results in an increase in LC_{50} of cytostatic drugs in patients with unmutated IgV_H genes
Cells were incubated as described under Fig. 5. For each cytostatic drug the LC_{50} was determined in the presence and absence of BCR triggering. Depicted is the relative change in LC_{50} (mean ± SEM) of the cytostatic drugs etoposide (A), chlorambucil (B) or fludarabine (C) as a consequence of BCR triggering for both unmutated (black bars) and mutated (white bars) IgV_H patients. The LC_{50} for each cytostatic drug increased after treatment of cells in combination with BCR ligation in unmutated IgV_H patients rendering these patients less sensitive for cytostatic drug-induced apoptosis.
B cell survival in IgV_H unmutated B-CLL patients

DISCUSSION

In the present study we have demonstrated that IgV_H unmutated and mutated B-CLL patients differ as to the functional consequences of BCR triggering. In unmutated IgV_H CLL BCR triggering resulted in rescue of leukemic B cells from spontaneous apoptosis as well as in a considerably decreased in vitro sensitivity to cytostatic drugs.

Two different subgroups of B-CLL patients can be identified based on the IgV_H mutation status of the malignant B cells.\textsuperscript{53} Comparison of IgV_H unmutated and mutated CLL patients has revealed remarkable differences in overall survival.\textsuperscript{55,56} At present the mechanism responsible for this difference in biological behavior is not known. Recent studies have provided evidence that may be pertinent to this issue. The observation that the unmutated IgV_H gene status is associated with a gene expression profile characteristic for BCR signaling suggests that cells of these patients are undergoing constant BCR-mediated stimulation in vivo.\textsuperscript{59} However, the nature of this stimulus remains elusive. In addition, these unmutated IgV_H cells have been reported to express ZAP-70 mRNA, a member of the Syk-ZAP-70 tyrosine kinase family, normally expressed in T cells and natural killer cells and critical in TCR-mediated signaling.\textsuperscript{86,88} In more recent studies the expression of ZAP-70 in CLL B cells of patients with unmutated IgV_H genes has also been demonstrated at the protein level.\textsuperscript{85,215} Our data confirm the association between IgV_H mutation status and expression of ZAP-70 and we also found that this association was not absolute.\textsuperscript{85,215} In agreement with other observations questioning the correlation between IgV_H mutation status and CD38 expression,\textsuperscript{71,73,76} we did not find a correlation between CD38 expression and IgV_H mutation status, ZAP-70 expression and/or response to BCR triggering.\textsuperscript{85}

Of course the key question is whether ZAP-70 is functionally active in the unmutated IgV_H CLL cells. That this might indeed be the case is suggested by the recent paper by Chen and coworkers\textsuperscript{215} who showed that following BCR ligation, CLL B cells of patients of the unmutated IgV_H subgroup exhibited significantly increased tyrosine phosphorylation of cytosolic proteins, including ZAP-70 and Syk,\textsuperscript{215,216} and association of ZAP-70 with proteins of the BCR complex.\textsuperscript{215} However, it remains to be shown that ZAP-70 is really instrumental for the functional consequences of BCR triggering in CLL. In addition to ZAP-70, differences in Syk expression and phosphorylation have been suggested to be responsible for the difference in BCR responsiveness in B-CLL patients.\textsuperscript{95,216-97} Although in a previous study we found that in CLL B cells Syk protein expression was associated with BCR signaling capacity,\textsuperscript{36} in our present study no difference in Syk protein expression was observed between IgV_H unmutated and mutated CLL patients (data not shown). This is in agreement with recent findings by others, who found differences between both CLL groups as to phosphorylation but not as to expression levels of Syk.\textsuperscript{215,216}

In the present study we aimed to study the functional consequences of BCR triggering in relation to IgV_H mutation status of CLL. Whereas no differences were observed between IgV_H unmutated or mutated CLL patients as to spontaneous apoptosis or cytostatic drug-induced apoptosis only (Fig. 2 and 3), in unmutated IgV_H patients BCR ligation rescued leukemic cells from undergoing spontaneous apoptosis and diminished the sensitivity of these cells to cytostatic drugs (Fig. 4, 5 and 6). These data indicate that in unmutated IgV_H CLL BCR signaling interferes with the induction of apoptosis. Previously, anti-IgM ligation has been shown to result in either induction of apoptosis\textsuperscript{101} or survival of leukemic B cells.\textsuperscript{102} In both studies, however, the correlation
between the BCR response and IgV<sub>H</sub> mutation status has not been investigated, possibly explaining the apparent difference with our results.

The protective effect of BCR triggering in unmutated IgV<sub>H</sub> patients was clearly evident within the range of concentrations of cytostatic drugs achieved during in vivo chemotherapeutic treatment of patients<sup>220-222</sup>. The in vitro protective effect of BCR triggering was only abolished at drug concentrations which by far exceed the toxic level in vivo<sup>220</sup>. Our results indicate that in unmutated IgV<sub>H</sub> patients chemotherapy might be less effective due to constant in vivo BCR signaling. At present there are no clinical data allowing conclusions as to treatment results in relation to IgV<sub>H</sub> mutation status of either de novo or relapsed B-CLL patients. Results of ongoing risk adapted CLL treatment trials will be of great importance.

Our study is the first to provide functional data on the effect of BCR triggering in IgV<sub>H</sub> unmutated and mutated B-CLL patients. The finding that in unmutated IgV<sub>H</sub> B-CLL patients BCR triggering protects from spontaneous apoptosis and reduces the sensitivity to cytostatic drug-induced apoptosis might explain the unfavorable clinical outcome in this subpopulation of CLL patients. Moreover, insight into the exact mechanism of the BCR-mediated responses might provide the basis for new targeted treatment modalities.

ACKNOWLEDGMENTS

The authors are indebted to the patients for their blood donations and to technicians from the department of Hematology, Academic Medical Center for technical assistance with patient material.